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(54) A method of DNA amplification.

(57) This invention relates to a method of amplifying template DNA by the PCR reaction wherein (a) a single oligonucleotide is contacted with said template DNA whereby said oligonucleotide randomly anneals to a single strand of said template DNA and DNA sequences complementary to the said single strand are synthesized using said oligonucleotide as a primer and said oligonucleotide also randomly anneals to the complementary strands whereby DNA sequences corresponding to the template DNA are synthesised so that PCR amplification yields synthetic DNA sequences which each incorporate said oligonucleotide sequence at the 5'end, and the complement of said oligonucleotide sequence at the 3'end, and (b) said synthesised DNA sequences then being subjected to further PCR amplification using said oligonucleotide as a primer.

It is possible by means of the method of this present invention to amplify trace quantities of DNA fragments of unknown sequence simply and efficiently, which is useful in the construction of DNA libraries of chromosome specific regions and the development of probes for their physical mapping.

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Figure 3 is an agarose electrophoresis pattern of DNA fragments tested with the method of this present invention.

In the present invention, the DNA sequence which is the subject of the amplification is a DNA fragment for which base sequence information has not been obtained (at least, a DNA fragment for which the base sequence information required for conventional PCR amplification has not been obtained), such as a chromosome specific region which has been physically cut out or a DNA sequence which has been cloned for a YAC library etc. for example. In the past, the ligation PCR method as described earlier could be used in cases where such DNA fragments were PCR amplified, but the method of this present invention is such that DNA fragments of unknown sequence can be amplified very effectively using more or less the same procedure and apparatus as used for conventional PCR without carrying out a chemical or enzymatic pre-treatment of the template DNA like that in the case of the ligation PCR method.

The method of the present invention involves first annealing a single type of oligonucleotide in an arbitrary region of a strand of the template DNA which has been thermally denatured, then extending the DNA chains which are complementary to the template DNA using this oligonucleotide as a PCR primer, and synthesising the complementary DNA corresponding to a random sequence region of the template DNA. Thus, by repeating the cycle of denaturing, annealing and extension, various DNA sequences having the primer sequence at one end and a sequence complementary thereto at the other end thereof corresponding to a random region of the template DNA may be synthesised (this process is referred to hereinafter as low stringency PCR).

It will be appreciated that the single oligonucleotide also anneals to the complementary strand to enable PCR amplification to take place.

Low stringency PCR differs from ordinary PCR in that it uses as a primer a single oligonucleotide of which the whole sequence is unlikely to be complementary to any sequence of the template DNA. A single primer of 20-30 nucleotides which has a plurality of restriction enzyme recognition sequences and which has at least two bases on the 3'-end which do not form restriction enzyme sites may be used. Thus, since random base sequences exist in the sequence of the DNA chains, the sequence of the 3'-end of the above mentioned nucleotide can provide a PCR primer in any region of the template DNA by annealing to complementary sequences on the template DNA. Furthermore, in cases where the above mentioned oligonucleotides are used as primers, the various DNA sequences which are ultimately amplified have a plurality of restriction enzyme cleavage sites at both ends and ligation to a vector etc. can be carried out easily. The synthetic nucleotide comprised of the base sequence 5'-TAGATCTGATATCTGAATTCCC-OH3' (referred to hereinafter as BVE22cc) can be cited as an actual example of such an oligonucleotide. BVE22cc has within the sequence comprised of 22 nucleotides recognition sequences for the restriction enzymes BglII, EcoRV and EcoRI (AGATCT, GATATC, GAATTC respectively) and it has a continuous sequence of cytosine (C) at the 3'- end. The DNA sequence which is amplified by means of the present invention can be used directly as a probe for the screening of genome libraries for example, and in this case it is also possible to use oligonucleotides which do not include a restriction enzyme recognition sequence.

However, as mentioned above, the low stringency PCR of this present invention involves the use of a oligonucleotide of which the complementary sequence for the template DNA is small and so the efficiency of the annealing of the template DNA and the primer is low.

Thus, with the method of the present invention it is recommended that the annealing reaction of the template DNA and the oligonucleotide in the low stringency PCR is carried out over a long period of time. That is to say, whereas the annealing reaction in ordinary PCR is of the order of 1 - 2 minutes, in the present invention it is set at 90 - 150 minutes and the opportunity for the template DNA and the primer to approach and hybridise is increased.

Furthermore, this annealing reaction is preferably carried out at low temperature. Ultimately, with ordinary PCR, the template DNA and the primer are annealed in a reaction solution at 50 - 60°C, but in such a temperature range the free primer and template DNA in the reaction solution are subject to the effects of thermal vibration and so mutual annealing is difficult even when they approach. Hence, the temperature of the reaction solution during the annealing reaction is set to a low temperature of 10 - 40°C and the effect of thermal vibration is minimised.

Moreover, in the method of the present invention an amphipathic polymer such as polyethylene glycol (PEG) or a polysaccharide such as glucose or sucrose for example may be added in order to increase the annealing efficiency of the template DNA and the primer. Ultimately, these amphipathic polymers increase the viscosity of the reaction solution and suitably suppress the brownian motion of the free template DNA and primer and increase the unification potential, providing conditions which are suitable for annealing. Moreover, these amphipathic polymers have no chemical action on the DNA molecule and no effect on the denaturation and extension steps in the PCR.

These means of increasing the annealing efficiency in low stringency PCR can be adopted suitably either

PstI fragment of the λ phage DNA (referred to hereinafter as λ/PstI) used as a size standard.

As is clear from Figure 1, in the method of the present invention the DNA fragment of unknown sequence cut out from the chromosome specific region (chromosome 4p 50 - 100%; Lane 1) was amplified with good efficiency in the same way as the RsaI fragment of human genome DNA (Lane 3 and 4). Moreover, the average sizes of the respective amplification products were about 500 bp for the human genome DNA fragment, and a smaller size of about 300 - 400 bp for the chromosome DNA fragment, implying that there is some mechanical or chemical damage during chromosome preparation. Furthermore, faint bands were obtained on amplifying the sublimated chromosome (all burned down metaphase; Lane 2) and in the absence of a template (no template; Lane 5), but these were thought to be due to inadequate sublimation treatment (laser irradiation) of the chromosome or to originate from some contamination in the manipulation process for example. In any case, there is a clear difference on comparing the amplification efficiency in the presence of the proper template DNA.

Example 2

The PCR products of the chromosome DNA obtained in Example 1 were each cloned in a λ phage vector and a library was built up.

First of all, the DNA was extracted from about half of the 3rd PCR solution in Example 1 by extraction with phenol/chloroform and chloroform/isoamyl alcohol, and by ethanol precipitation, and this DNA was treated with EcoRI to create cloning sites on both ends. After removing the very small DNA fragments by ultra-filtration, the fraction of amplified DNA (50 - 100 ng) was introduced and ligated to the EcoRI sites of λ phage gt11 which had been pre-treated with phosphatase and this was packaged in vitro and used to infect E. coli Y1090r cells. As a result 5×10^6 clones were obtained.

Next, 68 clones were selected randomly from this λ phage library and the inserts characterised. That is to say, the insert was amplified by means of conventional PCR taking each clone as template DNA and using a primer of the same sequence as the EcoRI part of the λ phage and the sequence adjacent thereto, and the size was analyzed by means of agarose electrophoresis.

The agarose electrophoresis patterns for 20 of the 68 types of insert analyzed are shown in Figure 2. The results showed that the insert size of the 68 clones analyzed was distributed over about 100 - 1500 bp, and this is in close agreement with the size distribution of the amplified chromosome DNA in Example 1 (see Figure 1). Furthermore, when these inserts were hybridised with human genome DNA (EcoRI or HindIII fragments), 40 - 60% of the clones were confirmed as being a human unique sequence, about 10% were confirmed as being a high frequency repeat sequence and 20 - 30% were confirmed as being a low - intermediate frequency repeat sequence.

Example 3

The optimum concentration range of the primer and/or amphipathic polymer in each PCR was tested.

10fg of RsaI digested human genome DNA was amplified in the same way as described in Example 1. The 1st PCR was carried out with a PEG concentration of 0, 8 or 12% in the reaction solution and with a primer (BVE22cc) concentration 0.2, 0.5 or 1.0 μM. After completing six PCR series, one sixth of each reaction solution was subjected to electrophoresis on 1.5% agarose, stained with ethidium bromide and the respective reaction efficiencies were analyzed.

The results of this experiment were as shown in the agarose electrophoresis patterns (1st PCR) of Figure 3. Moreover, in Figure 3 (1st PCR), Lane 1 shows the λ/PstI used as a size standard, and Lanes 2 - 4 show the states of amplification of the DNA fragment (10fg) with a primer concentration of 1.0 μM and PEG concentrations of 0, 8 and 12% respectively, while Lanes 5 - 7 show the states of amplification of the DNA fragment (10fg) with a PEG concentration of 12% and primer concentrations of 0.2, 0.5 and 1.0 μM respectively. As is clear from Figure 3 (1st PCR), the efficiency of the DNA chain synthesis increased as the PEG concentration was increased when the primer concentration was set at 1.0 μM (Lanes 2 - 4), and the efficiency of the synthesis increased as the primer concentration was increased when the PEG concentration was set at 12% (Lanes 5 - 7). However, although not shown in Figure 3, the synthesis of DNA chains was also observed in cases where no template DNA was present with a PEG concentration of more than 15% or a primer concentration of more than 3 μM. Consequently, it was adjudged that when BVE22cc is used for the primer and PEG is used for the amphipathic polymer in the low stringency PCR of this present invention, a PEG concentration in the reaction solution set within the range 10 - 15% and a primer concentration set within the range 1.0 - 1.5 μM is desirable.

Next, the solution which contained 12% PEG and 1.0 μM primer from among the various reaction solutions

Claims

1. A method of amplifying template DNA by the PCR reaction wherein (a) a single oligonucleotide is contacted with said template DNA whereby said oligonucleotide randomly anneals to a single strand of said template DNA and DNA sequences complementary to the said single strand are synthesized using said oligonucleotide as a primer and said oligonucleotide also randomly anneals to the complementary strands whereby DNA sequences corresponding to the template DNA are synthesised so that PCR amplification yields synthetic DNA sequences which each incorporate said oligonucleotide sequence at the 5'end and the complement of said oligonucleotide sequence at the 3'end, and (b) said synthesised DNA sequences then being subjected to further PCR amplification using said oligonucleotide as a primer.

2. A method of DNA amplification as claimed in Claim 1 wherein in step (a) the template DNA and the oligonucleotide are annealed for 90 - 150 minutes.

3. A method of DNA amplification as claimed in Claim 1 or Claim 2 wherein the template DNA and oligonucleotide are annealed at 10 - 40°C.

4. A method of DNA amplification as claimed in any one of the preceding claims wherein the template DNA and oligonucleotide are annealed in a reaction solution which contains an amphipathic polymer.

5. A method of DNA amplification as claimed in Claim 4 wherein the amphipathic polymer is polyethylene glycol of molecular weight 1,000 - 10,000.

6. A method of DNA amplification as claimed in any one of the preceding claims wherein the oligonucleotide is a single 20 - 30 base pair nucleotide which has a plurality of restriction enzyme recognition sequences and which has at least two bases at the 3' end which do not form restriction enzyme sites.

7. A method of DNA amplification as claimed in any one the preceding claims wherein the oligonucleotide has the following base sequence:

5′-TAGATCTGATATCTGAATTCCC-OH3′

8. A gene library in which DNA sequences amplified by the method of Claims 1 to 7 are cloned.

9. A method of characterising a target DNA molecule of unknown sequence wherein said DNA is amplified by the method of Claims 1 to 7 and sequenced.

10. A kit for amplifying target DNA by the method as claimed in claim 1 comprising:
    a) a single unique oligonucleotide;
    b) a polymerase; and
    c) nucleoside triphosphates.

Fig. 2